METHODS AND COMPOSITIONS FOR DELIVERING PROTEINS TO MACROPHAGE CELLS AND CELLS OF MACROPHAGE DERIVED LINEAGE

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FIELD OF THE INVENTION

The present invention relates to vaccines, methods for prophylactically and/or therapeutically immunizing individuals against immunogens, gene therapy compositions and methods of prophylactically and/or therapeutically treating individuals by delivering proteins to such individuals using gene therapy. This application claims priority to U.S. Provisional Application Serial No. 60/088,980 which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

Methods for delivering proteins to cells by direct DNA administration have been reported using a variety of protocols. Examples of such methods are described in U.S. Patent No. 5,593,972, U.S. Patent No. 5,739,118, U.S. Patent No. 5,580,859, U.S. Patent No. 5,589,466, U.S. Patent No. 5,703,055, U.S. Patent No. 5,622,712, U.S. Patent No. 5,459,127, U.S. Patent No. 5,676,954, U.S. Patent No. 5,614,503, and PCT Application PCT/US95/12502, which are each incorporated herein by reference.

Intramuscular injection of DNA expression cassettes leads to the *in vivo* expression of encoded proteins and has been shown to induce specific cellular and humoral immune responses. The intracellular expression of plasmids leads to the production of biologically active proteins which can be secreted and presented to B-cells facilitating the induction of an antibody response. Secreted proteins may also be ingested by professional

antigen presenting cells (APC), degraded within cellular endosomes and expressed on Major Histocompatability Complex (MCH) class II molecules. Transport of these peptide-MHC complexes to the cell surface facilitates antigen presentation to helper T-cells. The helper T-cell response and subsequent production of cytokines is a critical step in establishing an immune response. Additionally, finite quantities of all intracellularly produced proteins, including those encoded by DNA expression cassettes, are proteolysed endogenously and the resultant peptides loaded onto newly synthesized MHC class I molecules. Transport of these complexes to the cell surface is needed for the induction of a CTL response.

While potential pathogens may enter an animal through any number of routes, the central tenets of immunology support the induction of an immune response only at the secondary lymphoid tissues. Their architecture, location and ability to provide the cytokines and costimulatory signals needed to activate lymphocytes seem to suggest the secondary lymphoid organs as the most appropriate site for recognition and presentation of antigen. It is generally accepted that the majority of antigen in the blood is processed for antigen presentation in the spleen and antigens in tissue are transported and then processed and presented in the lymph nodes. Studies with DNA expression cassettes indicate that transfection and protein expression are primarily confined to the peripheral tissue. This raises the question of whether these transfected cells also have the ability to prime T-lymphocytes. Experiments suggest that T-lymphocyte induction and activation in the context of MHC-I is not restricted by transfected peripheral cells but rather by resident bone marrow derived APC. Thus, while transfected somatic tissues may serve as reservoirs of antigen, the initiation of T-lymphocyte responses occurs only after the transfer of antigen to professional bone marrow derived APC. Early results indicate that the activation of $CD8^+$ T-cells depends on these bone marrow derived professional APC.

The APC involved in the induction of the immune response following DNA immunization has also been the focus of attention. It seems appropriate that the induced immune response would be mediated by transfected APC at the site of inoculation. Indeed the migration of transfected skin dendritic cells (DC) to the lymph nodes following cutaneous delivery of plasmid delivery into the skin was reported recently. However, the contribution of this small number of transfected cells in immune induction is unclear. The ability of dendritic cells to prime immune responses after the direct administration of

retroviral vectors using a variety of antigen systems has been demonstrated. Dendritic cells might also prime immune responses following intramuscular plasmid delivery, however, the relatively low ratio of DC in muscle as compared to skin may limit this mechanism as the major mechanism for immune activation following intramuscular DNA inoculation.

While current vaccines can be effective to immunize individuals prophylactically or therapeutically against pathogen infection or human diseases, there is a need for improved vaccines. There is a need for compositions and methods which produce an enhanced immune response.

SUMMARY OF THE INVENTION

The present invention relates to methods of delivering a protein to a macrophage cell of an individual. The method comprises the step of administering to the individual at a site on the individual's body, a DNA molecule that comprises a nucleotide sequence that encodes the protein operably linked to a promoter that is functional in a macrophage cell and a polyadenylation signal that is functional in a macrophage cell. The DNA molecule is taken up by a macrophage cell and the nucleotide sequence is expressed to produce the protein in the macrophage cell.

The present invention further relates to methods of delivering a protein to a lymphnode of an individual. The methods comprise the steps of locating a site on said individual's body that is proximal to said lymphnode, and administering to the individual at that site, a DNA molecule comprising a nucleotide sequence that encodes the protein. The nucleotide sequence is operably linked to a promoter that is functional in a macrophage cell and a polyadenylation signal that is functional in a macrophage cell. The DNA molecule is taken up by a macrophage cell and the nucleotide sequence is expressed to produce the protein in the macrophage cell. The macrophage cell drains to the lymphnode, and delivers the protein in the lymphnode.

The present invention further relates to methods of inducing an immune response against an immunogen an individual. The methods comprise the step of administering to the individual at a site on the individual's body, a DNA molecule comprising a nucleotide sequence that encodes the immunogen. The nucleotide sequence is operably linked to a promoter that is functional in macrophage cells and a polyadenylation

signal that is functional in macrophage cells. The DNA molecule is taken up by a macrophage cell where the nucleotide sequence is expressed to produce the immunogen in the macrophage cell and an immune response mediated by the macrophage is generated against the immunogen.

The present invention further relates to methods of modulating an individual's immune system. The methods comprise the step of administering to the individual at a site on the individual's body, a DNA molecule comprising a nucleotide sequence that encodes an immunomodulating protein. The nucleotide sequence is operably linked to a promoter that is functional in a macrophage cell or a cell of macrophage derived lineage and a polyadenylation signal that is functional in a macrophage cell or a cell of macrophage derived lineage. The DNA molecule is taken up by a macrophage cell or a cell of macrophage derived lineage where the nucleotide sequence is expressed to produce the immunomodulating protein in the macrophage cell or a cell of macrophage derived lineage, and the immunomodulating protein modulates the individual's immune system.

The present invention further relates to methods of delivering proteins to an individual's macrophage cells and/or cells of macrophage derived lineage. The methods comprise the step of administering to the individual at a site on the individual's body, a DNA molecule comprising a nucleotide sequence that encodes an protein. The nucleotide sequence is operably linked to a promoter that is functional in a macrophage cell or a cell of macrophage derived lineage and a polyadenylation signal that is functional in a macrophage cell or a cell of macrophage derived lineage. The DNA molecule is taken up by a macrophage cell or a cell of macrophage derived lineage where the nucleotide sequence is expressed to produce the protein in the macrophage cell or a cell of macrophage derived lineage.

The present invention relates to methods of eliminating cells in a lymphnode of an individual. The methods comprise the step of administering to the individual at a site on said individual's body proximal to a lymphnode, a DNA molecule comprising a nucleotide sequence that encodes a cytotoxic protein. The nucleotide sequence is operably linked to promoter that is functional in macrophage cells and cells of macrophage derived lineages and a polyadenylation signal that is functional in macrophage cells and cells of macrophage derived lineages. The DNA molecule is taken up by macrophage cells or cells

of macrophage derived lineages and the nucleotide sequence is expressed to produce the protein in the macrophage cell or a cell of macrophage derived lineage. The macrophage cell or a cell of macrophage derived lineage drains to the lymphnode and secretes or releases the cytotoxic protein in the lymphnode, so that cells in the lymphnode are eliminated.

The present invention relates to pharmaceutical compositions for delivering proteins to macrophage cells or cells of macrophage derived lineages and to lymphnodes. The pharmaceutical compositions comprise DNA molecules comprising nucleotide sequences that encode proteins operably linked to promoters and polyadenylation signals that are functional in macrophage cells and cells of macrophage derived lineage.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B and 1C show data representing kinetics of expression and the immune response elicited by plasmid DNA innoculation. Analysis of protein expression following delivery of plasmid DNA. Serum was collected from mice immunized with a construct pcGag/Pol or saline control and analyzed by capture ELISA. Results shown are in Figure 1A in pg/ml as determined by a standardization curve using recombinant Pr24. Humoral immune responses were determined by ELISA using serum collected from immunized mice. Results are shown in Figure 1B. T-lymphocyte responses were analyzed in a MLR with splenocytes isolated from control and immunized mice. Proliferation in response to stimulation with recombinant Pr24 was measured by thymidine-H^3 incorporation. Results are shown in Figure 1C.

Figures 2A-2L show results from FACS analysis of macrophages isolated from peripheral blood following DNA innoculation. B7 expression was analyzed on peripheral blood macrophages isolated from saline, bupivacaine, plasmid control and pcEnv immunized. CD80 positive macrophages appear as early as 3 days (Figure 2D) post inoculation in pcEnv immunized mice and peak 14 days after inoculation (Figure 2E). Expression of CD80 returns to pre-inoculation levels (similar to day 3) 28 days after inoculation (Figure 2F). CD86 positive macrophages were also monitored in mice receiving DNA expression cassettes. Macrophages form mice inoculated with the pcEnv plasmid showed increased expression of CD86 at day 14 (Figure 2K) when compared to pre-immunization levels; day 3 (Figure 2J) and day 28 (Figure 2L) levels. Control plasmid mice did not show changes over the course of the study, day 3 CD80

(Figure 2A), day 14 CD80 (Figure 2B) and day 28 CD80 (Figure 2C) show little change throughout the experiment. Similarly CD86 control groups day 3 (Figure 2G), day 14 (Figure 2H) and day 28 (Figure 2I) showed little change through the observation period.

Figures 3A-L show data from FACS analysis of T-lymphocyte subsets isolated from peripheral blood following DNA innoculation. The expression of CD28 on peripheral blood $CD4^+$ and $CD8^+$ cells from plasmid control and pcEnv immunized was analyzed. CD4/CD28 positive T-cells show small increases as early as 3 days (Figure 3D) post inoculation and peak 14 days after inoculation (Figure 3E) in pcEnv immunized mice. Expression of CD4 returns to pre-inoculation levels (similar to day 3) 28 days after inoculation (Figure 3F). CD8/CD28 positive T-cells were also monitored in mice receiving DNA expression cassettes. Mice inoculated with the pcEnv plasmid showed increased expression of CD28 on CD8 T-cells at day 14 (Figure 3K) as compared to pre-immunization levels; day 3 (Figure 3J) and day 28 (Figure 3L) after inoculation. Control plasmid mice did not show changes over the course of the study, day 3 CD4 (Figure 3A), day 14 CD4 (Figure 3B) and day 28 CD4 (Figure 3C) show little change throughout the experiment. Similarly CD8 control groups day 3 (Figure 3G), day 14 (Figure 3H) and day 28 (Figure 3I) showed little change through the observation period.

Figures 4A-1 to 4A-4 show data from experiments on expression of CD69 on naive T-lymphocytes stimulated by *in vivo* transfected cells. Macrophages were isolated from the peripheral blood of mice immunized with pEGFP-C1 or plasmid control 14 days post immunization. Naive T-lymphocytes were incubated with these macrophages. Expression of CD69 on $CD4^+$ T-lymphocytes at the 2:1 ratio after 1 day (first row; Figures 4A-1 and 4A-2) and 3 days (second row; Figures 4A-3 to 4A-4) of *in vitro* stimulation is shown. Expression of CD69 induced by macrophages isolated from vector control mice (first column; Figures 4A-1 to 4A-3) and pcEnv injected mice (second column; Figures 4A-2 to 4A-4) is shown.

Figures 4B-1 to 4B-4 shows data from experiments on expression of CD28 on naive T-lymphocytes stimulated by *in vivo* transfected cells. Macrophages were isolated from the peripheral blood of mice immunized with pEGFP-C1 or plasmid control 14 days post immunization. Naive T-lymphocytes were incubated with these macrophages. Expression of CD28 on $CD4^+$ T-lymphocytes at the 2:1 ratio after 1 day (first row; Figures 4B-1 and 4B-2) and 3 days (second row; Figures 4B-3 and 4B-4) of in vitro stimulation is shown. Expression of CD28 induced by macrophages isolated from vector control mice (first column; Figures 4B-1 and 4B-3) and pcEnv injected mice (second column; Figures 4B-2 and 4B-4) is shown.

Figure 5 shows data from experiments studying *in vitro* proliferation of naive T-lymphocytes primed by macrophages and stimulated with recombinant protein. Macrophages sorted from the peripheral blood of mice immunized with pcEnv expression cassettes and plasmid control were incubated with naive T-lymphocytes at a 2:1 ratio, in the presence of thymidine-H^3. T-cell thymidine-H^3 incorporation following stimulation with transfected macrophages in the presence of recombinant protein is shown. Polyclonal PHA stimulation gave $>10^5$ CPM.

Figure 6 illustrates the migration of transfected macrophages. Macrophages and muscle are transfected following plasmid inoculation. Transfected macrophages are activated and migrate to the draining lymph node for antigen presentation. Some macrophages return to the blood lymphocyte pool and are distributed to other organs, however these cells are capable of priming T-lymphocyte responses while in the peripheral blood.

Figure 7 shows a diagram of the pNeZCD3α.1 expression plasmid. The components of this plasmid include the murine CD3-δ sequences necessary for specific expression in immune cells.

Figures 8A and 8B show data from experiments from Example 2. Spleen size induced by *Nef* antigen with and without immunomodulatory proteins expressed under the control of CMV and CD3 promoters is shown. Nef-WT refers to constructs with Nef under the control of the CMV promoter. IP-1 and IP-2 refers to refers to constructs with Nef under the control of the CD3 promoter.

Figure 9 shows data from experiments from Example 2. These data are the antibody responses induced by *nef* if different expression vectors.

DESCRIPTION OF PREFERRED EMBODIMENTS

The invention arises from the discovery that macrophage take up DNA administered by direct DNA administration and that such transfected macrophage migrate to lymphnodes. Accordingly, direct DNA administration of DNA constructs which include regulatory elements that function in macrophages and cells of macrophage derived lineage can be used to deliver proteins to macrophage cells and cells of macrophage derived lineage and lymphnodes.

The relationships between the *in vivo* transfected cells following direct intramuscular inoculation and immune induction have been defined and the kinetics of immune activation following delivery of DNA expression cassettes has been examined. The observed kinetics were correlated with the expression and distribution of cell surface proteins. Particular cell surface proteins whose changes on T-lymphocytes and APC in the peripheral blood appear to correlate with the kinetics of the immune response were used to study the cells important in immune activation.

Importantly, transfected macrophages expressing activation markers and T-cell co-stimulatory ligands were present in the peripheral compartment as well as lymph nodes of mice. These macrophages directly activate T-lymphocytes *in vitro* which confirms their ability to present antigen (Ag) and function as primary APC. Macrophages are known to function as APC, thereby providing cytokines for the activation of T-cells. Macrophages also express the co-stimulatory molecules CD80 and CD86 which play a dominant role in T cell activation. This study demonstrates that APC in the blood lymphocyte pool, peripheral tissue and lymphoid organs are activated following DNA inoculation. Further these cells may play an important role in T-lymphocyte activation through antigen presentation and co-ligation of the T cell receptor complex.

As used herein the term "macrophage promoter and promoter specific for cells of macrophage derived lineage" is meant to refer to a promoter that encodes a protein endogenously produced by macrophage cells and/or cells of macrophage derived lineage. Examples of such promoters include promoters for any proteins expressed in macrophages and cells of macrophage derived lineage including CD3, CD4, the CD11 antigens (such as CD11 A, CD11 B and CD11 C), CD12, CD13, CD14, CD15, CD16, CD17, CD21, CD23, CD25, CD26, CD30, CD31, CD32, CD33, CD36, CD39, CD40, CD45RO, CD45RA, CD45RB, CD49A, CD49B, CD49D, CD49E, CD49F, CD50, CD57, CD60, CD61, CD62L, CD63, CD64, CD65, CD68, CD69, CD70, CD74, CD80, CD84, CD85, CD86, CD87, CD88, CD89, CD91, CD92, CD93, CD97, CD101, CD102, CD105, CD114, CD115 (MCSFreceptor), CD119, CD121B, CD127, CD135, CD148, CD155, CD156, CD157, CD163, proteins involved in the maintenance of homeostasis in the cell, proteins involved in cell motion including actin, cellular adhesion molecules, chemokines (RANTES, MIP1α, MIP1β, MDC, TARK), and molecules involved in the immune system (MHC-I, MHC-II,

etc.). Macrophage promoters also includes promoters for any proteins expressed specifically in macrophages and/or cells of macrophage derived lineage including catalase, CD156, M-CSFR, p73, and FcgRI.

As used herein the term "macrophage-specific promoter and/or and promoter specific for cells of macrophage derived lineage" is meant to refer to a promoter that encodes a protein endogenously produced exclusively by macrophage cells and/or cells of macrophage derived lineage. Examples of such promoters include promoters for proteins expressed specifically in macrophages such as catalase, CD156, M-CSFR, p73, and FcgRI.

As used herein the term "delivers said protein in said lymphnode" is meant to refer to the secretion or other release of said protein by a macrophage into said lymphnode.

As used herein the term "an immune response mediated by said macrophage and cells of macrophage derived lineage" is meant to refer to an immune response initiated or potentiated by the presentation of antigens or the release of immuno-stimulatory proteins by macrophage cells and cells of macrophage derived lineage.

As used herein the term "protective immune response" is meant to refer to an immune response which targets an immunogen to which the individual has not yet been exposed such as a pathogen antigen in an uninfected individual, or a disease cell associated protein in an individual who does not have the disease such as a tumor associated protein in a patient who does not have a tumor.

As used herein the term "therapeutic immune response" is meant to refer to an immune response which targets an immunogen to which the individual has been exposed such a pathogen antigen in an infected individual, or a disease cell associated protein in an individual who has the disease such as a tumor associated protein in a patient who has a tumor.

As used herein the term "prophylactically effective amount" is meant to refer to the amount necessary to, in the case of infectious agents, prevent an individual from developing an infection, and in the case of cell specific diseases, prevent an individual from developing a cell specific disease.

As used herein the term "therapeutically effective amount" is meant to refer to the amount necessary to, in the case of infectious agents, reduce the level of infection in an infected individual in order to reduce symptoms or eliminate the infection, and in the case

of cell specific diseases, reduce the number of cell specific disease cells in an individual with a cell specific disease in order to reduce symptoms or cure the individual.

As used herein the term "cell specific disease" is meant to refer to autoimmune diseases and diseases characterized by hyperproliferating cells such as cancer.

As used herein, the term "immunomodulating proteins" is meant to refer to proteins and nucleic acid molecule expression products which enhance and/or modulate the immune response. Accordingly, immunomodulating proteins may be delivered as immunotherapeutics or as components in a vaccine.

As used herein the term "a site on said individual's body proximal to a lymphnode" is meant to refer to a location from which macrophage cells or cells of macrophage derived lineage drain from the site to a particular lymphnode.

As used herein the term "cytotoxic protein" is meant to a protein which kills cells such as a toxin, or a protein which has a cytostatic effect on cells.

According to the present invention, methods of delivering protein to macrophage cells and cells of macrophage derived lineage of an individual are provided. Such methods comprise administering to the individual at a site on the individual's body, a DNA molecule comprising a nucleotide sequence that encodes the protein to be delivered. The nucleotide sequence that encodes the protein is operably linked to a promoter that is functional in a macrophage cell or a cell of macrophage derived lineage and a polyadenylation signal that is functional in a macrophage cell or a cell of macrophage derived lineage. When the DNA molecules are administered to the individual, they are taken up by macrophage cells and cells of macrophage derived lineage and the nucleotide sequence that encodes the protein is expressed, producing the protein in the macrophages and cells of macrophage derived lineage.

Depending upon the nature of the protein so produced, various aspects of the present invention, such as those set forth below, can be exploited. For example, by using macrophage to express DNA administered by direct DNA administration, protein may be delivered to lymphnodes. If the protein encoded by the DNA molecule is an immunogen, the expression of the protein in macrophage provides methods of inducing immune responses in individuals. If the protein encoded by the DNA molecule is an immunomodulating protein, the expression of the protein in macrophage provides methods

of modulating the immune systems of individuals. If the protein encoded by the DNA molecule is a toxin, the expression of the protein in macrophage provides methods of eliminating cells in a lymphnode. In each and similar instances, as for example described in more particular detail below, there are certain preferred embodiments.

According to some methods of the invention, the DNA molecule is administered by a route of administration selected from the group consisting of: intramuscularly, intranasally, intraperatoneally, subcutaneously, intradermally, or topically or by lavage to mucosal tissue selected from the group consisting of vaginal, rectal, urethral, buccal and sublingual. Preferred routes of administration include intradermal, subcutaneous, intraperitoneal, intramuscular, and oral.

According to some methods of the invention, the DNA molecule is a plasmid.

According to some methods of the invention, the promoter is a macrophage promoter such as promoters for any proteins expressed in macrophages, including proteins involved in the maintenance of homeostasis in the cell, any proteins involved in cell motion including actin, and cellular adhesion molecules, including CD11, CD13, and molecules involved in the immune system (MHC-I, MHC-II, CD25, CD80, CD86 etc.). In some embodiments, the promoter is a macrophage-specific promoter such as promoters for catalase, CD156, M-CSFR, p73, and FcγRI

According to some methods of the invention, the promoter is selected from the group consisting of the CD3 promoter, CMV promoter, Actin promoter, MHC promoter, an SV40 promoter, and a Malony virus promoter.

According to some methods of the invention, the polyadenylation signal is selected from the group consisting of an SV40 polyadenylation signal and bovine growth hormone polyadenylation signal.

According to some methods of the invention, the DNA molecule is administered with a composition which facilitates uptake of DNA molecules by a cell. In some embodiments, the nucleic acid molecule is delivered to the cells in conjunction with the administration of a co-agent. Examples of co-agents are described in U.S. Patent No. 5,593,972, U.S. Patent No. 5,739,118 and International Application Serial Number PCT/US94/00899 filed January 26, 1994, which are each incorporated herein by reference. The co-agents which are administered in conjunction with nucleic acid molecules may be administered as a mixture

with the nucleic acid molecule or administered separately simultaneously, before, or after administration of nucleic acid molecules. In some embodiments, co-agents may be cationic lipids, including but not limited to, those described in U.S. Patent No. 5,703,055. Examples of other co-agents include growth factors, cytokines and lymphokines such as α-interferon, gamma-interferon, platelet derived growth factor (PDGF), TNF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12 as well as fibroblast growth factor, surface active agents such as immune-stimulating complexes (ISCOMS), Freund's incomplete adjuvant, LPS analog including monophosphoryl Lipid A (MPL), Cholera toxin, cobra toxin, saponins, muramyl peptides, quinone analogs and vesicles such as squalene and squalene, and hyaluronic acid. In some embodiments, an immunomodulating protein may be used as a co-agent. Preferred compositions that facilitate uptake of DNA molecule by a cell are selected from the group consisting of: cationic lipids, liposomes and local anesthetics. In some preferred embodiments, the DNA molecule is administered with bupivacaine. In some embodiments, multiple co-agents are used. The co-agents which are administered in conjunction with nucleic acid molecules may be administered as a mixture with the nucleic acid molecule or administered separately simultaneously, before or after administration of nucleic acid molecules.

One aspect of the present invention provides methods of delivering protein to a lymphnode of an individual. The present invention provides the means to deliver proteins to them by administering DNA at a site from where macrophages that take up such DNA will drain. The first step in delivering protein to a lymphnode of an individual is to locate a site on said individual's body that is proximal to the lymphnode to which delivery of the protein is intended. Sites are chosen for delivery to lymphnodes based on the anatomical draining pattern.

Once the site has been located, the DNA is administered to the individual at that site. The DNA molecule comprises a nucleotide sequence that encodes the protein to be delivered to the lymphnode and is operably linked to a promoter that is functional in a macrophage cell or a cell of macrophage derived lineage and a polyadenylation signal that is functional in a macrophage cell or a cell of macrophage derived lineage. When the DNA molecule is taken up by macrophage cells and cells of macrophage derived lineage, the nucleotide sequence is expressed to produce the protein in the macrophage cell and cells of macrophage

derived lineage. The macrophage cells and cells of macrophage derived lineage drain to the lymphnode and deliver the protein in the lymphnode. The protein is delivered to the lymphnode for example by secretion or destruction/death of the macrophage and release of the cell contents in the lymphnode. In some preferred embodiments, the protein comprises a secretion signal sequence to facilitate secretion from the macrophage. In some preferred embodiments, the protein delivered to the lymphnode is an anti-infective agent. In some preferred embodiments, the protein delivered to the lymphnode is a therapeutic protein such as a growth factor.

According to another aspect of the present invention, methods of inducing an immune response against an immunogen in an individual are provided. Such methods comprise the step of administering to the individual at a site on said individual's body, a DNA molecule comprising a nucleotide sequence that encodes the immunogen operably linked to a promoter that is functional in macrophage cell or a cell of macrophage derived lineages and a polyadenylation signal that is functional in macrophage cells and cells of macrophage derived lineage. The DNA molecule is taken up by a macrophage cell or a cell of macrophage derived lineage where said nucleotide sequence is expressed to produce the immunogen in the macrophage cell or a cell of macrophage derived lineage. An immune response mediated by the macrophage is generated against the immunogen.

The genetic material is expressed by the individual's cells and serves as an immunogenic target against which an immune response is elicited. The resulting immune response is broad based: in addition to a humoral immune response, both arms of the cellular immune response are elicited. The methods of the present invention are useful for conferring prophylactic and therapeutic immunity. Thus, a method of immunizing includes both methods of immunizing against immunogens and thus for example of protecting an individual from pathogen challenge, or occurrence or proliferation of specific cells as well as methods of treating an individual suffering from pathogen infection, hyperproliferative disease or autoimmune disease.

As used herein the term "target protein" is meant to refer to peptides and proteins encoded by gene constructs of the present invention which act as target proteins for an immune response. The term "target protein" and "immunogen' are used interchangeably and refer to any protein against which an immune response can be elicited. The target protein

is preferably an immunogenic protein which shares at least an epitope with a protein from the pathogen or undesirable cell-type such as a cancer cell or a cell involved in autoimmune disease against which immunization is required. The immune response directed against the target protein will protect the individual against and/or treat the individual for the specific infection or disease with which the target protein is associated.

The present invention is useful to elicit broad immune responses against a target protein, i.e. proteins specifically associated with pathogens, allergens or the individual's own "abnormal" cells. The present invention is useful to immunize individuals against pathogenic agents and organisms such that an immune response against a pathogen protein provides protective immunity against the pathogen. The present invention is useful to combat hyperproliferative diseases and disorders such as cancer by eliciting an immune response against a target protein that is specifically associated with the hyperproliferative cells. The present invention is useful to combat autoimmune diseases and disorders by eliciting an immune response against a target protein that is specifically associated with cells involved in the autoimmune condition.

Nucleic acid molecules which are delivered to macrophage cells and cells of macrophage derived lineage according to the invention may serve as genetic templates for immunogens that function as prophylactic and/or therapeutic immunizing agents. The present invention may be used to immunize an individual against all pathogens such as viruses, prokaryotes and pathogenic eukaryotic organisms such as unicellular pathogenic organisms and multicellular parasites. The present invention is particularly useful to immunize an individual against those pathogens which infect cells and which are not encapsulated such as viruses, and prokaryote such as gonorrhoea, listeria and shigella. In addition, the present invention is also useful to immunize an individual against protozoan pathogens which include a stage in the life cycle where they are intracellular pathogens. As used herein, the term "intracellular pathogen" is meant to refer to a virus or pathogenic organism that, during at least part of its reproductive or life cycle, exists within a host cell and therein produces or causes to be produced, pathogen proteins. Table 1 provides a listing of some of the viral families and genera for which vaccines according to the present invention can be made. DNA constructs that comprise DNA sequences which encode the peptides that comprise at least an epitope identical or substantially similar to an epitope

displayed on a pathogen antigen such as those antigens listed on the tables are useful in vaccines. Moreover, the present invention is also useful to immunize an individual against other pathogens including prokaryotic and eukaryotic protozoan pathogens as well as multicellular parasites such as those listed on Table 2.

In order to produce a genetic vaccine to protect against pathogen infection, genetic material which encodes immunogenic proteins against which a protective immune response can be mounted must be included in a genetic construct as the coding sequence for the target. Whether the pathogen infects intracellularly, for which the present invention is particularly useful, or extracellularly, it is unlikely that all pathogen antigens will elicit a protective response. Because DNA and RNA are both relatively small and can be produced relatively easily, the present invention provides the additional advantage of allowing for vaccination with multiple pathogen antigens. The genetic construct used in the genetic vaccine can include genetic material which encodes many pathogen antigens. For example, several viral genes may be included in a single construct thereby providing multiple targets.

Tables 1 and 2 include lists of some of the pathogenic agents and organisms for which genetic vaccines can be prepared to protect an individual from infection by them. In some preferred embodiments, the methods of immunizing an individual against a pathogen are directed against HIV, HTLV or HBV.

Another aspect of the present invention provides a method of conferring a broad based protective immune response against hyperproliferating cells that are characteristic in hyperproliferative diseases and to a method of treating individuals suffering from hyperproliferative diseases. As used herein, the term "hyperproliferative diseases" is meant to refer to those diseases and disorders characterized by hyperproliferation of cells. Examples of hyperproliferative diseases include all forms of cancer and psoriasis.

Introduction of a genetic construct that includes a nucleotide sequence which encodes an immunogenic "hyperproliferating cell"-associated protein into the macrophage of an individual results in the production of those proteins in the macrophage of the vaccinated individual. As used herein, the term "hyperproliferative-associated protein" is meant to refer to proteins that are associated with a hyperproliferative disease. To immunize against hyperproliferative diseases, a genetic construct that includes a nucleotide sequence which

encodes a protein that is associated with a hyperproliferative disease is administered to an individual.

In order for the hyperproliferative-associated protein to be an effective immunogenic target, it must be a protein that is produced exclusively or at higher levels in hyperproliferative cells as compared to normal cells. Target antigens include such proteins, fragments thereof and peptides which comprise at least an epitope found on such proteins. In some cases, a hyperproliferative-associated protein is the product of a mutation of a gene that encodes a protein. The mutated gene encodes a protein which is nearly identical to the normal protein except it has a slightly different amino acid sequence which results in a different epitope not found on the normal protein. Such target proteins include those which are proteins encoded by oncogenes such as *myb, myc, fyn,* and the translocation gene *bcr/abl, ras, src,* P53, *neu, trk* and EGRF. In addition to oncogene products as target antigens, target proteins for anti-cancer treatments and protective regimens include variable regions of antibodies made by B cell lymphomas and variable regions of T cell receptors of T cell lymphomas which, in some embodiments, are also used as target antigens for autoimmune disease. Other tumor-associated proteins can be used as target proteins such as proteins which are found at higher levels in tumor cells including the protein recognized by monoclonal antibody 17-1A and folate binding proteins.

While the present invention may be used to immunize an individual against one or more of several forms of cancer, the present invention is particularly useful to prophylactically immunize an individual who is predisposed to develop a particular cancer or who has had cancer and is therefore susceptible to a relapse. Developments in genetics and technology as well as epidemiology allow for the determination of probability and risk assessment for the development of cancer in individual. Using genetic screening and/or family health histories, it is possible to predict the probability a particular individual has for developing any one of several types of cancer.

Similarly, those individuals who have already developed cancer and who have been treated to remove the cancer or are otherwise in remission are particularly susceptible to relapse and reoccurrence. As part of a treatment regimen, such individuals can be immunized against the cancer that they have been diagnosed as having had in order to combat a recurrence. Thus, once it is known that an individual has had a type of cancer and

is at risk of a relapse, they can be immunized in order to prepare their immune system to combat any future appearance of the cancer.

The present invention provides a method of treating individuals suffering from hyperproliferative diseases. In such methods, the introduction of genetic constructs serves as an immunotherapeutic, directing and promoting the immune system of the individual to combat hyperproliferative cells that produce the target protein.

The present invention provides a method of treating individuals suffering from autoimmune diseases and disorders by conferring a broad based protective immune response against targets that are associated with autoimmunity including cell receptors and cells which produce "self"-directed antibodies.

T cell mediated autoimmune diseases include Rheumatoid arthritis (RA), multiple sclerosis (MS), Sjogren's syndrome, sarcoidosis, insulin dependent diabetes mellitus (IDDM), autoimmune thyroiditis, reactive arthritis, ankylosing spondylitis, scleroderma, polymyositis, dermatomyositis, psoriasis, vasculitis, Wegener's granulomatosis, Crohn's disease and ulcerative colitis. Each of these diseases is characterized by T cell receptors that bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of the T cells would elicit an immune response including CTLs to eliminate those T cells.

In RA, several specific variable regions of T cell receptors (TCRs) which are involved in the disease have been characterized. These TCRs include Vβ-3, Vβ-14, Vβ-17 and Vα-17. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in RA. See: Howell, M.D., *et al.*, 1991 *Proc. Natl. Acad. Sci. USA* **88**:10921-10925; Paliard, X., *et al.*, 1991 *Science* **253**:325-329; Williams, W.V., *et al.*, 1992 *J. Clin. Invest.* **90**:326-333; each of which is incorporated herein by reference.

In MS, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include Vβ-7 and Vα-10. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in MS. See: Wucherpfennig, K.W., *et al.*, 1990 *Science* **248**:1016-1019; Oksenberg, J.R., *et al.*, 1990 *Nature* **345**:344-346; each of which is incorporated herein by reference.

In scleroderma, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include Vβ-6, Vβ-8, Vβ-14 and Vα-16, Vα-3C, Vα-7, Vα-14, Vα-15, Vα-16, Vα-28 and Vα-12. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in scleroderma.

In order to treat patients suffering from a T cell mediated autoimmune disease, particularly those for which the variable region of the TCR has yet to be characterized, a synovial biopsy can be performed. Samples of the T cells present can be taken and the variable region of those TCRs identified using standard techniques. Genetic vaccines can be prepared using this information.

B cell mediated autoimmune diseases include Lupus (SLE), Grave's disease, myasthenia gravis, autoimmune hemolytic anemia, autoimmune thrombocytopenia, asthma, cryoglobulinemia, primary biliary sclerosis and pernicious anemia. Each of these diseases is characterized by antibodies which bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of antibodies would elicit an immune response including CTLs to eliminate those B cells that produce the antibody.

In order to treat patients suffering from a B cell mediated autoimmune disease, the variable region of the antibodies involved in the autoimmune activity must be identified. A biopsy can be performed and samples of the antibodies present at a site of inflammation can be taken. The variable region of those antibodies can be identified using standard techniques. Genetic vaccines can be prepared using this information.

In the case of SLE, one antigen is believed to be DNA. Thus, in patients to be immunized against SLE, their sera can be screened for anti-DNA antibodies and a vaccine can be prepared which includes DNA constructs that encode the variable region of such anti-DNA antibodies found in the sera.

Common structural features among the variable regions of both TCRs and antibodies are well known. The DNA sequence encoding a particular TCR or antibody can generally be found following well known methods such as those described in Kabat, *et al.* 1987 *Sequence of Proteins of Immunological Interest* U.S. Department of Health and Human Services, Bethesda MD, which is incorporated herein by reference. In addition, a general

method for cloning functional variable regions from antibodies can be found in Chaudhary, V.K., *et al.*, 1990 *Proc. Natl. Acad. Sci. USA* **87**:1066, which is incorporated herein by reference.

In some preferred embodiments, the method of the invention comprises administering a DNA molecule that encodes an immunogen, and additionally comprises a nucleotide sequence that encodes an immunomodulating protein. The nucleotide sequence that encodes the immunomodulating protein is operably linked to a promoter that is functional in macrophage cells and cells of macrophage derived lineage and a polyadenylation signal that is functional in macrophage cells and cells of macrophage derived lineage. In other preferred embodiments, two DNA molecules are administered to the individual's body. The second DNA molecule comprises a nucleotide sequence that encodes an immunomodulating protein operably linked to a promoter that is functional in macrophage cells and a polyadenylation signal that is functional in macrophage cells. According to either of these preferred embodiments, the immunomodulating protein improves or otherwise enhances the immune response generated by the individual. In some embodiments, the immunomodulating protein is selected from the group consisting of chemokines, IL-12, B7.1, B7.2, CD40, LFA, cytokines and lymphokines such as α-interferon, gamma-interferon, platelet derived growth factor (PDGF), TNF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFNγ, IL-18, IL-5 and other cytokines. In some embodiments, it is preferred that the gene for GM-CSF is included in genetic constructs used in immunizing compositions.

In some preferred embodiments, in addition to an immunogen, proteins that assist the macrophage in antigen presentation are delivered to cells using DNA molecules that encode them. The coding sequences are operably linked to a promoter that is functional in macrophage cells and cells of macrophage derived lineage and a polyadenylation signal that is functional in macrophage cells and cells of macrophage derived lineage. In some embodiments, the protein is a CD4 molecule.

In some preferred embodiments, in addition to an immunogen, proteins that assist the macrophage in antigen presentation are delivered to cells using DNA molecules that encode them in handing antigens off to dendritic cells. The phenomenon in which macrophage normally hand antigens off to dendritic cells occurs when macrophage cell or a cell of

macrophage derived lineage death is occurring. Accordingly, by providing macrophage with expressible forms of DNA that encodes both an immunogen and a cell death signal, transfer of the immunogen or a fragment thereof to a dendritic cell can be facilitated. The cell death signals include, for example, Fas, trad, FADD, traf, ICE and related Caspaces, TNFα, and toxins such as ricin A chain. The coding sequences of the death signals are operably linked to a promoter that is functional in macrophage cells and cells of macrophage derived lineage and a polyadenylation signal that is functional in macrophage cells and cells of macrophage derived lineage. Other proteins which can be used to facilitate handing off of antigen from macrophage and cells of macrophage derived lineage to dendritic cells include proteins that are attractants for dendritic cells. Accordingly, by providing macrophage and cells of macrophage derived lineage with expressible forms of DNA that encodes both an immunogen and an attractants for dendritic cells, transfer of the immunogen or a fragment thereof to a dendritic cell can be facilitated. Attractants for dendritic cells include, for example, chemokines such as RANTES, MCP, MDCCK1, MDCCK2 and MDCCK3 chain. The coding sequences of the attractants for dendritic cells are operably linked to a promoter that is functional in macrophage cells and cells of macrophage derived lineage and a polyadenylation signal that is functional in macrophage cells and cells of macrophage derived lineage.

Some aspects of the present invention relate to methods of modulating an individual's immune system that comprise administering to the individual at a site on their body, a DNA molecule that comprises a nucleotide sequence that encodes an immunomodulating protein. The nucleotide sequence that encodes the immunomodulating protein is operably linked to a promoter that is functional in a macrophage cell or a cell of macrophage derived lineage and a polyadenylation signal that is functional in a macrophage cell or a cell of macrophage derived lineage. When the DNA molecule is taken up by a macrophage cell or a cell of macrophage derived lineage, the nucleotide sequence is expressed to produce the immunomodulating protein in said macrophage cell or a cell of macrophage derived lineage and the immunomodulating protein modulates the individual's immune system. In some embodiments, the immunomodulating protein results in an expansion of the number of macrophages. In some embodiments, the immunomodulating protein results in an reduction in the number of macrophages, shutting down the immune response. In some embodiments,

the immunomodulating protein is selected from the group consisting of growth factors, chemokines, IL-12, B7.1, B7.2, CD40, LFA, IL-2, IFNγ, IL-18, IL-5 and other cytokines.

According to some aspects of the present invention, methods of delivering proteins to an individual's macrophage cells and/or cells of macrophage derived lineage are provided. The methods comprise the step of administering to the individual at a site on the individual's body, a DNA molecule comprising a nucleotide sequence that encodes an protein. The nucleotide sequence is operably linked to a promoter that is functional in a macrophage cell or a cell of macrophage derived lineage and a polyadenylation signal that is functional in a macrophage cell or a cell of macrophage derived lineage. The DNA molecule is taken up by a macrophage cell or a cell of macrophage derived lineage where the nucleotide sequence is expressed to produce the protein in the macrophage cell or a cell of macrophage derived lineage. The protein may be any protein for which delivery to the individual is desired.

According to some aspects of the invention, methods are provided for eliminating cells in a lymphnode of an individual without surgical intervention. Often, such as in the case where malignant cells are found in lymphnode biopsies, surgical intervention carries the risk of resulting in a spread of cancer due to physical limitations in the removal of all cells during resection. According to the invention, delivery of a cytotoxic agent to the lymphnodes using methods of the present invention can eliminate cells in the lymphnode and, in some cases the entire lymphnode, without the risk of spreading malignancy associated with surgical resection. The methods comprise administering to the individual at a site on said individual's body proximal to the lymphnode, a DNA molecule comprising a nucleotide sequence that encodes cytotoxic protein operably linked to promoter that is functional in macrophage cells and cells of macrophage derived lineage and a polyadenylation signal that is functional in macrophage cells and cells of macrophage derived lineage. The DNA molecule is taken up by macrophage cell or cells of macrophage derived lineages and the nucleotide sequence is expressed to produce the protein in the macrophage cell or a cell of macrophage derived lineage. The macrophage cell or a cell of macrophage derived lineage drains to the lymphnode, and secretes or otherwise releases the cytotoxic protein in the lymph node eliminating cells in lymphnode. As discussed above, the lymphnodes into which a macrophage drains, i.e. the proximal lymphnode, can be selected and the site where administration of DNA for such delivery can be located. When

the DNA molecule administered to the site is taken up by macrophage cells or cells of macrophage derived lineages, the nucleotide sequence is expressed to produce the protein in the macrophage cells or cells of macrophage derived lineage. The macrophage cell or a cell of macrophage derived lineages drain to the lymphnode and deliver the protein in the lymphnode. The protein is delivered to the lymphnode for example by secretion or destruction/death of the macrophage and release of the cell contents in the lymphnode. In preferred embodiments, the cytotoxic protein comprises a secretion signal sequence. In preferred embodiments, the protein is a toxin. In preferred embodiments, the protein is a toxin selected from the group consisting of: ricin A chain, and diphtheria toxin.

As used herein, the term "genetic construct" refers to the DNA molecules that comprise a nucleotide sequence which encodes the protein and which includes initiation and termination signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the macrophage cells and cells of macrophage derived lineage of the individual.

As used herein, the term "expressible form" refers to gene constructs which contain the necessary regulatory elements that are functional in macrophage and cells of macrophage derived lineage and operably linked to a coding sequence that encodes a protein, such that when present in the cell of the individual, the coding sequence will be expressed.

Methods that comprise direct DNA administration are particularly described in the U.S. Patents set forth above which have been incorporated by reference.

Briefly, genetic constructs may comprise a nucleotide sequence that encodes a protein operably linked to regulatory elements needed for gene expression. According to the invention, those regulatory elements must be functional in macrophage cells and cells of macrophage derived lineage. In preferred embodiments, the regulatory elements are derived from macrophage cells or cells of macrophage derived lineages, and in more preferred embodiments, the regulatory elements are specific for macrophage cells or cells of macrophage derived lineages.

When taken up by a macrophage cell or a cell of macrophage derived lineage, the genetic construct may remain present in the macrophage cell or a cell of macrophage derived lineage as a functioning extrachromosomal molecule and/or integrate into the cell's chromosomal DNA. DNA may be introduced into macrophage cells and cells of

macrophage derived lineage where it remains as separate genetic material in the form of a plasmid or plasmids. When introducing DNA into the macrophage cell or a cell of macrophage derived lineage, reagents which promote DNA integration into chromosomes may be added. DNA sequences which are useful to promote integration may also be included in the DNA molecule. It is also contemplated to provide the genetic construct as a linear minichromosome including a centromere, telomeres and an origin of replication.

Genetic constructs include regulatory elements necessary for gene expression of a nucleic acid molecule. The elements include: a promoter, an initiation codon, a stop codon, and a polyadenylation signal. These elements must be functional in macrophage cells and cells of macrophage derived lineage.

Initiation codons and stop codon are generally considered to be part of a nucleotide sequence that encodes the protein. However, it is necessary that these elements are functional in the macrophage of the individual to whom the gene construct is administered. The initiation and termination codons must be in frame with the coding sequence.

In addition to the regulatory elements required for DNA expression, other elements may also be included in the DNA molecule. Such additional elements include enhancers. The enhancer may be selected from the group including but not limited to: human Actin, human Myosin, human Hemoglobin, human muscle creatine and viral enhancers such as those from CMV, RSV and EBV.

Genetic constructs can be provided with mammalian origin of replication in order to maintain the construct extrachromosomally and produce multiple copies of the construct in the cell. Plasmids pCEP4 and pREP4 from Invitrogen (San Diego, CA) contain the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region which produces high copy episomal replication without integration.

An aspect of the present invention relates to pharmaceutical compositions useful in the methods of the present invention. The pharmaceutical compositions comprise a DNA molecule comprising a nucleotide sequence that encodes protein operably linked to a promoter that is functional in macrophage cells and cells of macrophage derived lineage and a polyadenylation signal that is functional in macrophage cells and cells of macrophage derived lineage; and a pharmaceutically acceptable carrier or diluent.

The pharmaceutical compositions according to the present invention comprise about 1 ng to about 10,000 μg of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 2000 μg, 3000 μg, 4000 μg or 5000 μg of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 1000 μg of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 10 ng to about 800 μg of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 0.1 to about 500 μg of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 1 to about 350 μg of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 25 to about 250 μg of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 100 μg DNA.

The term "pharmaceutical" is well known and widely understood by those skilled in the art. As used herein, the terms "pharmaceutical compositions" and injectable pharmaceutical compositions" are meant to have their ordinary meaning as understood by those skilled in the art.

The pharmaceutical compositions according to the present invention are formulated according to the mode of administration to be used. One having ordinary skill in the art can readily formulate a vaccine that comprises a genetic construct. In cases where intramuscular injection is the chosen mode of administration, an isotonic formulation is preferably used. Generally, additives for isotonicity can include sodium chloride, dextrose, mannitol, sorbitol and lactose. In some cases, isotonic solutions such as phosphate buffered saline are preferred. Stabilizers include gelatin and albumin. In some embodiments, a vasoconstriction agent is added to the formulation. The pharmaceutical preparations according to the present invention are provided sterile and pyrogen free.

In a preferred embodiment, the DNA is administered by intramuscular injection. Bupivacaine, a well known and commercially available pharmaceutical compound, is administered prior to, simultaneously with or subsequent to the genetic construct. Bupivacaine and the genetic construct may be formulated in the same composition. Bupivacaine is particularly useful in view of its many properties and activities when administered to tissue. Bupivacaine is related chemically and pharmacologically to the aminoacyl local anesthetics. It is a homologue of mepivacaine and related to lidocaine.

Bupivacaine renders muscle tissue voltage sensitive to sodium challenge and effects ion concentration within the cells. A complete description of bupivacaine's pharmacological activities can be found in Ritchie, J. M. and N. M. Greene, The Pharmacological Basis of Therapeutics, Eds.: Gilman, A.G. et al, 8th Edition, Chapter 15: 3111, which is incorporated herein by reference. Bupivacaine and compounds that display a functional similarity to bupivacaine are preferred in the method of the present invention.

Bupivacaine-HCl is chemically designated as 2-piperidinecarboxamide, 1-butyl-N-(2,6-dimethylphenyl)monohydrochloride, monohydrate and is widely available commercially for pharmaceutical uses from many sources including Astra Pharmaceutical Products Inc. (Westboro, Mass.) and Sanofi Winthrop Pharmaceuticals (New York, N.Y.). Bupivacaine is commercially formulated with and without methylparaben and with or without epinephrine. Any such formulation may be used. It is commercially available for pharmaceutical use in concentrations of 0.25%, 0.5% and 0.75% which may be used on the invention. Alternative concentrations which elicit desirable effects may be prepared if desired. According to the present invention, about 250 μg to about 10 mg of bupivacaine is administered. In some embodiments, about 250 μg to about 7.5 mg is administered. In some embodiments, about 0.50 mg to about 5.0 mg is administered. In some embodiments, about 1.0 mg to about 3.0 mg is administered. In some embodiments about 5.0 mg is administered. For example, in some embodiments about 50 μl to about 2 ml, preferably 50 μl to about 1500 μl and more preferably about 1 ml of 0.5% bupivacaine-HCl and 0.1% methylparaben in an isotonic pharmaceutical carrier is administered at the same site as the vaccine before, simultaneously with, or after the vaccine is administered. Similarly, in some embodiments, about 50 μl to about 2 ml, preferably 50 μl to about 1500 μl and more preferably about 1 ml of 0.5% bupivacaine-HCl in an isotonic pharmaceutical carrier is administered at the same site as the vaccine before, simultaneously with, or after the vaccine is administered. Bupivacaine and any other similarly acting compounds, particularly those of the related family of local anesthetics, may be administered at concentrations which provide the desired facilitation of uptake of genetic constructs by cells.

In some embodiments of the invention, the individual is first subjected to bupivacaine injection prior to genetic vaccination by intramuscular injection. That is, for example, up to about a week to ten days prior to vaccination, the individual is first injected with

bupivacaine. In some embodiments, prior to vaccination, the individual is injected with bupivacaine about 1 to 5 days before administration of the genetic construct. In some embodiments, prior to vaccination, the individual is injected with bupivacaine about 24 hrs before administration of the genetic construct. Alternatively, bupivacaine can be injected simultaneously, minutes before or after vaccination.

Accordingly, bupivacaine and the genetic construct may be combined and injected simultaneously as a mixture. In some embodiments, the bupivacaine is administered after administration of the genetic construct. For example, up to about a week to ten days after administration of the genetic construct, the individual is injected with bupivacaine. In some embodiments, the individual is injected with bupivacaine about 24 hrs after vaccination. In some embodiments, the individual is injected with bupivacaine about 1 to 5 days after vaccination. In some embodiments, the individual is administered bupivacaine up to about a week to ten days after vaccination.

The present invention may be performed using local anesthetics as facilitators. In addition to bupivacaine, mepivacaine, lidocaine, procaine, carbocaine and methyl bupivacaine, other similarly acting compounds may be used.

EXAMPLES

Example 1

Materials and Methods

Plasmids and Reagents.

Protein expression was analyzed using the pcGag/Pol DNA expression cassette encoding the HIV-1 core Protein (Pr55) and protease enzyme under CMV promoter control. It has been shown that transfection of COS-7 cells with this plasmid leads to the production and secretion of the cleavage product, Protein 24 ($Pr24^{)}$. The cellular events associated with immune activation were studied with the DNA expression cassette pcEnv, encoding the HIV-1 gp160 envelope protein under CMV promoter control. Controls for these experiments included inoculations of (a) normal saline, (b) bupivicaine solution and © vector control. Localization of plasmid and studies of tissue distribution were accomplished with the pEGFP-C1 plasmid (Clontech Laboratories Inc., Palo Alto CA). This plasmid encodes a red-shifted-mutant green fluorescent protein with peak excitation at 488nm.

Immunization with plasmid expression cassettes.

Female Balb/c mice 4-6 wk. old were obtained from Charles River Laboratories Inc., Wilmington, MA. All animals used in this study were maintained at the University of Pennsylvania, Philadelphia under supervision of ULAR. The animals received 50 μg doses of each DNA expression cassette. All plasmid DNA was formulated to 1 mg/ml in PBS-0.25% Bupivacaine-HCl (1-Butyl-N-2,6-dimethylphenyl-2-piperidinecarboxamide) (Astra Pharmaceutical Products, Inc., Westborough MA) and delivered by intramuscular injection into the right quadriceps muscle in at least two separate sites.

Detection of protein expression in vivo.

Protein expression was tested by ELISA. Mice were immunized with pcGag/Pol as described; animals were bled and antisera collected and pooled. One hundred microliters of serum and serum dilutions of 1:10, 1:50 and 1:100 were analyzed by capture ELISA using the Coulter HIV-1 Pr24 Antigen Assay (Coulter, Miami Fl.)

Detection of antibody titres.

Animals were inoculated and antisera collected as described previously. Fifty microliters of recombinant Pr55 (Quality Biological, Inc., Gaithersburg, MD) were diluted in 0.1 M Carbonate-Bicarbonate buffer (pH 9.5) to a final concentration of 2 μg/ml and absorbed for 1 hr. at room temperature onto 96-well microtitre enhanced protein binding plates. The plates were washed twice with PBS-0.05% Tween-20 and blocked with 5% Non-Fat Dry Milk, 0.05% Tween-20 in PBS for 1 hr. at 37°C. Mouse antisera was diluted with PBS and incubated on plates for 1 hr. at 37°C. The plates were washed and incubated with HRP-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO); then washed and developed with 3,3',5,5'-TMB solution. Optical density at 450 nm was read on a Dynatech MR5000 spectrophotometer.

Flow Cytometry.

Animals were inoculated as previously described. At the time of harvest mice were anesthetized with an intraperitoneal injection of 300 μL AVERTIN (5 gm 2-2-2 Tribromoethanol (Aldrich, Milwaukee WI) in 5 ml Tert-amyl-alcohol (Aldrich, Milwaukee WI) 50X soln.). Peripheral blood was collected by cardiocentesis into heparinized blood collection tubes (20 U/ml). Following blood collection the right inguinal lymph node was removed and spleen harvested. Spleens and lymph nodes were crushed in a tissue shredder

to remove excess tissue and release cells. For flow cytometry analysis, Peripheral Blood Mononuclear Cells(PBMC) were isolated from blood by washing 3-4 times in 1X Geys solution to remove erythrocytes. Following isolation, samples were washed twice in Fluorescein Activated Cell Sorting (FACS) Buffer (PBS-1% BSA, 0.1% NaN_3) then blocked for 15 min at 4°C in FACS Buffer supplemented with 10% Goat Serum (Sigma, St. Louis MO). Cells were then resuspended to a concentration of 5 x 10^6 cells/ml, and 100 µL cells incubated with antibody combinations for 1 hr at 4°C. Unbound antibody was removed by washing in FACS buffer. Samples were analyzed using a Coulter EPICS XL-MCL flowcytometer. Antibodies specific to the following cell surface proteins were used in the analysis: CD4 and CD8 (Sigma), B220 , MAC-3 , MAC-1 , Ly-49C , CD28 , CTLA-4 , CD80 and CD86 , CD40 and CD40L (Pharmingen).

Sorting and Immunofluorescence of PBMC.

PBMC were collected and treated as described in FACS assays. Sodium azide was excluded from buffers to facilitate cell culture after sorting. After incubation with appropriate antibodies, cells were washed and resuspended in RPMI 1640, 5% FBS growth media. Cells were sorted on Coulter EPICS ELITE and collected in RPMI 1640, 50% FBS. After sorting, cells were allowed to sit for 30 minutes then pelleted and resuspended in RPMI 1640, 50% FBS. Cells were placed into chamber slides and photographed with appropriate red and green filters on a Nikon Diophot microscope.

Immunohistochemistry and Immunofluorescence of tissue samples.

Muscle samples from the quadriceps at the DNA inoculation site, inguinal lymph nodes and spleen were removed for immunohistochemistry and immunofluorescence. Samples were fixed in PBS-2% paraformaldehyde for 4 hrs, washed in 1X PBS and incubated in PBS-5% sucrose for 2 hrs. Samples were cryoprotected in PBS-20% sucrose, shock frozen and sectioned. Tissue sections were incubated with biotinylated antibodies specific to mouse CD80 and CD86 at 1µg/10^6 cells (Pharmingen). Slides were then incubated with Avidin-Texas Red (Vector, Burlingame, CA) for 1 hr at 4°C and photographed.

T-helper cell proliferation assay.

Lymphocytes were harvested from as previously described. The isolated splenocytes were then resuspended to a concentration of 1 x 10^6 cells/ml. A 100 µl aliquot containing

1×10^5 cells was immediately added to each well of a 96 well microtitre round bottom plate. Recombinant Pr55 protein at the final concentration of 5 μg/ml and 0.5 μg/ml was added to wells in triplicate. The cells were incubated at 37°C in 5% CO_2 for three days. One microcurie of thymidine-H^3 was added to each well and the cells were incubated for 12 to 18 hours at 37°C. The plate was harvested and the amount of incorporated tritiated thymidine was measured in a Beta Plate reader (Wallac, Turku, Finland). PHA stimulation was used in this experiment as a polyclonal activator and positive control.

Analysis of APC activity in Macrophages.

To determine the ability of macrophages to stimulate T-lymphocytes *in vitro*, groups of mice inoculated with pcEnv and plasmid control were compared. Macrophages were isolated by FACS using antibodies specific to the Mac-1 and Mac-3 antigen from both groups of mice 14 days post inoculation. Concurrently lymphocytes were separated from the spleen of naive mice and naive T-lymphocytes further isolated by panning with mouse IgG specific antibody (Sigma). The efficiency of T-lymphocyte separation was tested by flow cytometry using a monoclonal antibody to CD3 (Sigma). Naive T-lymphocytes and macrophages were incubated at ratios of 10:1, 4:1 and 2:1. Cells were harvested after three days of stimulation, stained and analyzed by flow cytometry. Macrophages and T-lymphocytes were incubated together for one day, followed by the addition of recombinant protein for three days in a proliferation experiment. Cells were harvested and analyzed as previously described in T-helper cell proliferation assay.

Results

The relation between protein antigen expression and immune induction by DNA vaccines is not yet clear. Accordingly, we evaluated the relationship between antigen expression and the development of humoral and cellular immune responses.

Protein Expression.

The kinetics of protein expression following inoculation with pcGag/Pol was tested by ELISA. Mice were immunized with 50 μg pcGag/Pol plasmid DNA and plasmid control as described in Materials and Methods. Serum was collected on days 0, 1, 2, 3, 4, 5, 6, 9, 14 and 30 post inoculation and tested for the presence of free Pr24 (HIV-1 core proteins). Protein expression followed the kinetics usually seen during in vitro transfection. Following a short lag phase, Pr24 was detected in the serum of mice two days after inoculation. Protein

levels increased then peaked at day 5-6; levels then decreased slowly in a roughly exponential manner (Figure 1A). Pr24 could not be detected in the serum of naive and plasmid control mice; these group showed little reactivity throughout the study.

Humoral Immune Response.

Humoral immune responses were detectable in the experimental mice following plasmid delivery. Mice inoculated with pcGag/Pol DNA expression cassettes developed observable Pr24 specific antibodies as early as 7 days after a single inoculation. The antibody response continued to rise through day 30 (Figure 1B). The rise in anti-Pr24 antibody level accompanied a concurrent fall in free Pr24 levels detectable in the serum of the immunized mice, suggesting that the immune response may have inhibited our ability to detect Pr24 in the serum. The control mice did not develop humoral response to Pr24 during the study.

T-helper Response.

T-lymphocyte proliferation responses were also analyzed in animals which received the pcGag/Pol plasmid DNA. Mice were inoculated and spleens harvested every day for one week, then at two and four weeks post inoculation. T-lymphocytes incubated in a mixed lymphocyte reaction and stimulated with recombinant Pr55 show increased thymidine incorporation as compared to cells stimulated with an irrelevant antigen or the cells similarly stimulated from naive and plasmid control mice (Figure 1C). The result of the T-lymphocyte proliferation assay indicated that the cellular response follows a pattern similar to that of protein expression with a lag of some 8-9 days. Proliferation was not observed in the first 4 days post inoculation; increased on day 5; and reached a maximum on day 14 post inoculation. The levels of thymidine incorporation after Pr55 stimulation returned to normal levels by day 30, and was indistinguishable from control animals shortly thereafter. Control mice groups showed no response to Pr24 stimulation throughout the study. These results suggest that *in vivo* expression is transient and the duration of expression may have implications for immune boosting.

Screening of PBMC and Splenocytes.

To further characterize immune activation, splenocytes and PBMC were surveyed for changes in the expression of activation markers following inoculation with DNA expression cassettes. Multicolor FACS analysis with antibodies to distinguish cell subtype and

combinations of antibodies to known immune activation markers was performed. In initial experiments animals (n = 5) were inoculated with 50 μg pcEnv as described in Materials and Methods. Splenocytes and PBMC were examined for changes in cell surface markers 14 days post inoculation; mice were analyzed individually and the results averaged to determine the mean change.

Changes in the expression of activation markers are indexed by cell type and summarized in Tables 3A and 3B. On T-lymphocytes we examined CD28, a co-stimulatory signal and ligand for CD80 and CD86. CD40-L, the natural ligand for CD40, and a T-lymphocyte activation signal. CTLA-4, found on activated T-cells and involved in the downregulation of T-cells was also followed. An upregulation of CD28, CD40-L and CTLA-4 activation signals on both the $CD4^+$ and $CD8^+$ T-cells was observed. Macrophages in the peripheral blood showed increases in CD80 and CD86 expression (Table 3A) suggesting a relationship with APC ability. Further, the numbers of macrophages now expressing the co-stimulatory ligands represents a significant percent of the total macrophage pool. B-cells and NK isolated from the PBMC showed very small changes in the activation markers examined.

The changes in T-cells and macrophages observed in PBMC were not observed among the splenocytes. To ensure small changes in spleen T-cells and macrophages were not obscured by the large B-cell population in the spleen further experiments with purified $CD4^+$ and $CD8^+$ T-cells were done. With T-cells isolated from the spleen of pcEnv immunized mice the result of our survey did not change. Among splenocytes only B-cells show some activation in CD40 expression with changes of 5% or more in comparison to the control groups (Table 3B).

As described in Materials and Methods there were multiple controls to these experiments the most significant of which is the vector control. In the saline and bupivicaine control groups there were minimal changes at various time points. The vector control group was very similar to the previous control groups except for some time points where a 1-3% deviation from the saline control was observed.

Analysis of Cell Activation

Based on the changes noted in our initial survey, a more extensive analysis of T-lymphocytes and macrophages was designed to illustrate the relationship between the

induced immune response and changes in activation at the cellular level. Animals receiving 50 μg pcEnv and vector control were divided into three groups (n = 6) and analyzed at 3, 14, and 28 days post inoculation. PBMC and splenocyte samples were collected, pooled and analyzed by FACS as described in Materials & Methods. Following pcEnv immunization, an increase in the percentage of $CD3^+$ cells was observed at day 14 in the spleen and PBMC. Concurrent with the increase in $CD3^+$ cells, macrophages in the spleen and peripheral blood showed maximal CD80 and CD86 expression. Activated macrophages in the periphery increased by 10% with an increase of 5% in the spleen. In PBMC, following an initial decrease on day 3, macrophages that express CD86 returned to normal or increased by 0-5% by day 28 (Figures 2A-2L). Mice inoculated with control plasmid did not show changes in B7 expression patterns and are similar to naive.

An increase in the number of activated T-lymphocytes was observed following inoculation with pcEnv. CD8/CD28 positive PBMC increased by 15-20% and a 10% increase in the CD8/CTLA-4 cells present in the periphery was also observed (Table 3A). The increase in CD4/CD28 positive cells in the blood lymphocyte pool was 10-15% comparable to the 10% increase observed for CD4/CTLA-4 in the PBMC. The changes in the splenocyte population were less dramatic. There were minimal changes in the number of CD8/CD28, CD4/CD28, CD8/CTLA-4 and CD4/CTLA-4 expressing cells (Table 3B). The most dramatic population shifts occurred in the $CD28^+$ T-lymphocyte population as well as in B7 expression on macrophages (Figures 3A-3L). The significant T-cell activation observed 14 days post inoculation is in agreement with the results obtained in functional assays (Figure 1C) where maximum proliferation occurs at approximately the day 14 time point. This data also agrees with prior reports that DNA vaccines induce CTL which is present at day 14 following inoculation. Again, modulation of the activation signals was more easily demonstrated in the PBMC than the splenocyte population.

Identification of DNA Transfected Antigen Presenting Cells.

Green Fluorescent Protein (GFP) encoding vectors were used to determine if the observed activation of macrophages was due to the direct transfection of cells in vivo or due to endocytosis of secreted GFP. Intramuscular inoculation of the pEGFP-C1 plasmid leads to in vivo transfection and production of the GFP, which is detected by conventional fluorescence microscopy. The quadriceps muscle of Balb/c mice immunized with saline,

bupivicaine, control plasmid or GFP expressing plasmid were sectioned and photographed 14 days after immunization. In the muscle, expression of GFP follows the time course of expression observed for Pr24. The GFP expression vector was used as an immunogen with antibodies to detect activation markers expressed on transfected PBMC. GFP expression can be seen in myocytes 3-4 days post inoculation and is still present in isolated muscle fibers at day 14. No expression of CD80 or CD86 was observed on muscle fibers stained with specific Ab 14 days after inoculation. Samples from plasmid control groups showed no GFP expression or CD80 or CD86 staining.

Three to four days following immunization, very few scattered individual cells with GFP expression can also be seen in the spleen; however expression in spleen is undetectable 14 days after inoculation. Staining for CD80 and CD86 shows no co-expression of these proteins on the surface of these cells. In contrast to these results, GFP expression is detectable in the proximal lymph node at 3 and 14 days post inoculation. Within the proximal lymph node, but not in LN^{14} taken from plasmid control mice, transfected cells are arranged in small clusters in approx. 1-2 areas per lymph node. These transfected cells showed lower levels of CD80 expression and relatively high levels of CD86 expression. The frequency of green loci was calculated for mice inoculated with GFP. In general only the lymph node proximal to the site of inoculation showed significant GFP signal. Both experimental and plasmid control mice LN show areas of background CD80 and CD86 expression not associated with green loci. This is in agreement with previous reports of CD80 and CD86 expression in LN and spleen.

Macrophages were sorted from the pooled splenocytes and PBMC of animals immunized with 50 μg pEGFP-C1 and control plasmid fourteen days after inoculation. These cells were analyzed by immunofluorescence microscopy and transfected macrophages photographed. Among cells sorted from the spleen, no green cells were found by immunofluorescence indicating a lack of transfected cells in the spleen. In contrast, there were transfected/green cells observed in the peripheral blood. The cells were then restained and analyzed with anti-CD80 (Pharmingen) and anti-CD86 (Pharmingen) Ab. Importantly, these transfected macrophages from the peripheral blood compartment were found to express CD86.

Analysis of Macrophages as Antigen Presenting Cells.

Based on the evidence that there were transfected circulating activated macrophages with antigen presenting capability, we tested the ability of macrophages separated from pcEnv immunized mice to activate naive T-lymphocytes. Purified naive T-lymphocytes were incubated with macrophages separated from inoculated, naive and plasmid control mice. Activation was measured by FACS and proliferation assays. Activation was evident early in T-lymphocytes stimulated with macrophages from immunized mice. An increase in expression of CD69 (Very Early Activation Antigen) on $CD4^+$ cells and to a lesser extent on $CD8^+$ T-lymphocytes indicates the macrophage capacity to present Ag following DNA immunization (Figures 4A-1 to 4A-4). The VEAA molecule (CD69) is found at very low levels on all lymphocytes; enhanced expression is known to occur within hours of in vivo and in vitro activation of T-lymphocytes. CD28 expression which was unchanged at day 1 was observed to increase primarily on $CD4^+$ T-lymphocytes at day 3 (Figures 4B-1 to 4B-4). However, by day 3 CD69 expression had returned to preimmunization levels.

The functional relevance of these activated macrophages was further evaluated in a T-lymphocyte proliferation assay. Here, naive T-lymphocytes primed by activated macrophages derived from animals immunized with pcEnv showed significant incorporation of thymidine in response to incubation with recombinant gp120, while macrophages derived from plasmid control mice did not show this result (Figure 5). Polyclonal activation with PHA was used as a positive control in these assays. These data demonstrate that transfected APC which are activated and expressing increased levels of B7.2 can be important primary APC in immune activation following DNA vaccination. It is important to note that activation was only noted at a 2:1 ratio of T-lymphocytes to macrophages indicating the very small number of transfected macrophages obtained after immunization.

Discussion

Genetic immunization is an attractive method for inducing adaptive immunity. The induction of humoral and cell-mediated immune responses have been demonstrated following the delivery of plasmid DNA, through a number of routes, and via multiple delivery systems. Genetic immunization offers several advantages over traditional vaccination strategies including rapid construction and testing of vaccines. The technique of DNA immunization is likely safer than other live vaccination strategies. DNA expression cassettes pose little threat to the immunocompromised host. No significant immune

response against the expression cassette has been observed, and pathogenic or immunomodulatory elements can be removed from the plasmid further enhancing vector safety.

Currently our understanding of immune activation is incomplete. In this regard, understanding immune activation following DNA immunization may provide insight into immune mechanisms. This understanding may also aid in vaccine and immune therapeutic design. Recently, several groups have begun to investigate the mechanism(s) by which DNA expression cassettes induce immune responses. Early results indicate that this pathway is more complex than originally thought. Here we have investigated the changes in surface proteins related to immune activation following plasmid DNA immunization in an effort to define the events associated with immune activation and T-lymphocyte priming.

The kinetics of immune activation following plasmid inoculation indicate that protein expression precedes and drives immune activation (Figure 1A). The lag time observed between plasmid delivery and protein detection in the serum distinguishes the time of *in vivo* transfection from translation and secretion of encoded plasmid proteins. The humoral immune response further lagged protein expression by several days (Figure 1B). This is not surprising as it is not unreasonable to expect some minimum protein concentration is needed to surpass the activation threshold of B-cells and develop a functional helper T-cell response. T-cell proliferation after inoculation was present following antigen expression. Following transfection and antigen expression, specific T-lymphocyte activation occurs (Figure 1C) as evidenced by T-cell proliferation. The activation of $CD4^+$ T-lymphocytes peaks and falls in a pattern similar to the expression of antigen and coincident with the induction of antibody responses.

It is noteworthy that the antibody response did not diminish over the short observation period of this study. In studies with other vectors, the antibody response persisted for several months. This is in agreement with the results of several authors that have found persistent antibody responses following DNA inoculation. $CD8^+$ CTL was not analyzed in this study; previous reports have indicated that CTL activity is very low after one inoculation but appears 14 days following a boost, suggesting that the sensitivity of the assay would limit this initial study.

These results indicate both humoral and cellular responses appears to be coordinated with protein expression. Specific T-cell activation was further investigated: the T-cell subsets were studied in more detail by following the activation markers and costimulatory molecules on T-lymphocytes. The roles of the CD28 and CTLA-4 (CD152) proteins have recently become the focus of much investigation. Additionally, the role of CD40-L as another costimulatory molecule to T-lymphocytes has recently received attention. The expression of the costimulatory molecule CD28, both by mean florescence and percent positive cells, increased in both the $CD4^+$ and $CD8^+$ T-cell populations following DNA inoculation (Figure 3). Our results indicate that DNA immunization (a) increases the number of circulating T-lymphocytes expressing the CD28 molecule, and (b) increases expression of CD28 in both T-lymphocyte subsets. The elevation of CD28 expression has been linked to a stronger immune response and the development of resistance to HIV infection in human cells. Further, in experiments performed by our group, the development of a protective immune response to HIV-1 correlated with an increase in CD28 expression in the PBMC of immunized chimpanzees. The correlation of CD28 expression and induced immune responses indicates that this may be an important early predictor of the effectiveness of a particular immunization cassette.

Plasmid DNA also induced increased expression of CTLA-4 and CD40-L. CTLA-4 is known to be transiently expressed in T-lymphocytes following immune activation and is thought to provide a down-modulatory signal to T-lymphocytes. It is a powerful inhibitor of T-cells, CTLA-4 knockout mice exhibit lymphoproliferative disorders and develop fatal spontaneous autoimmune disease. The increased expression of CTLA-4 followed the same time course as CD28. It is possible that this competing ligand is induced to control the active T-cell or to suppress the threshold activation of other T-cell clones.

Another important finding clearly observed here is that the activation of T-cells by plasmid DNA inoculation is an inducible and transient phenomenon. The activation of T-cells requires specific TCR-MHC complexes in addition to a co-stimulatory signal provided by the B7 family of molecules. T-lymphocytes costimulated by B7 through the CD28 receptor and TCR complex in the absence of other stimulatory signals may upregulate pleiotropic immune activities. However, the expression of CD80 and CD86 on professional APC *in situ* in both normal and pathological tissues, and its up-regulation on monocytes by

GM-CSF and IFN-γ is consistent with this role of CD28 in driving T-cell stimulation. The data presented here supports that DNA antigens function *in vivo* through this cascade.

The co-stimulatory ligands followed a time-dependent expression pattern which mimicked that of the observed T-cell proliferation. The expression of CD80 and CD86 was observed to increase through day 14 then to decrease concurrent with T-cell activation. This result was also observed in the LN where the number of transfected cells expressing the B7 molecules increased after plasmid delivery but then decreased by day 10-14. Unexpectedly, there was also an increase in CD86 expression in the circulating cells by day 3. Among circulating cells the changes in B7.2 expression was almost entirely confined to macrophages. Interestingly these CD86 expressing macrophages are also transfected raising interesting questions as to the role of these circulating cells and their potential as APC in the periphery. Functional experiments using these transfected macrophages as APC show that these macrophages, transfected in vivo, can activate naive $CD4^+$ T-lymphocytes *in vitro*. Thus while there are functional APC in the peripheral blood, the role of these cells *in vivo* must be further analyzed.

The earliest reports on DNA immunization showed in vivo transfection and antigen presentation following gene delivery was mainly confined to the immediate somatic tissue. However, our improved understanding of cytokine and costimulatory molecule distribution makes the muscle an unlikely primary APC. Muscle APC activity is limited to MHC-I expression and we have found no evidence of B7 expression on muscle cells. The inefficiency of the muscle cell as APC was further demonstrated by Kim *et al.* In an investigation of the co-delivery of plasmids encoding the B7 molecules it was found that CD86 codelivery and expression increased $CD8^+$ T-lymphocyte restricted CTL responses to HIV-1 targets. In elegant studies by Doe *et al.* the importance of a second APC in plasmid DNA-induced immune responses was further reinforced. In adoptive transfer experiments in which bone marrow cells from a F1 $H2^d$ & $H2^k$ hybrid mouse were transplanted into $H2^d$ mice, it was reported that CTL to $H2^k$ restricted epitopes could be measured. These studies indicated the importance of a second bone marrow derived antigen presenting cell in priming T-lymphocyte responses. The identification of this second APC has become the focus of much attention. It is thought that this cell acts as an intermediary between the muscle and $CD8^+$ T-lymphocyte: engulfing antigen and presenting it on MHC-I

molecules, facilitating T-lymphocytes priming. The mechanism by which immunogens expressed by transfected myocytes are processed and presented as a peptide-MHC-I complex has not been fully elucidated.

The direct transfection of professional APC is another method by which antigens encoded by plasmid DNA could be presented on MHC-I molecules and prime $CD8^+$ T-lymphocytes. Plasmid in the muscle, lymph node, peripheral blood and spleen were observed following intramuscular inoculation of a GFP expressing plasmid. These transfected cells showed diffuse cytoplasmic GFP signal consistent with intracellular GFP expression as opposed to a localized vesicle pattern that would suggest phagocytosis of secreted GFP. GFP localization in the LN and spleen could be the result of direct transfection or the trafficking of transfected cells into these organ. In genetic immunization, the very short half-life of extracellular DNA in lymph and blood makes it unlikely that plasmids directly transfect the lymph node or spleen in significant numbers. However, studies of DNA elimination kinetics in the thyroid and synovial fluid indicate that plasmids may in general be long lived in tissue and interstitial fluids with a $t_{1/2}$ of 10 to 40 hr. The longevity of DNA expression cassettes in the tissue increases the likelihood that cells are transfected at the site of inoculation; these transfected cells then travel to other organs (Figure 6). It seems most likely that the localization of plasmid in the lymph node and spleen would be the result of immigrating transfected cells.

The migration of transfected dendritic cells to the lymph node following DNA immunization in the skin was recently shown. Transfected skin-derived dendritic cells can be detected in the lymph nodes 24 hours after immunization. While transfected dendritic cells may be important in intramuscular inoculation, the density of DC in muscle is expected to be lower than in skin. Transfected cells in the regional lymph node have been observed 14 days following intramuscular immunization. These cells have been identified as macrophages; they are transfected and, these small clusters of transfected cells express the B7 ligands necessary for T-cell activation. This result suggests that transfected macrophages at the site of immunization migrate to the lymph node following intramuscular inoculation and may be an important APC, possibly playing a role similar to the bone marrow APC.

Macrophages express both MHC class I and II and the co-stimulatory ligands for T-lymphocyte activation. In these experiments, transfected macrophages have the necessary

capabilities to prime both antibody and cell-mediated immune responses. The transfection of macrophages is itself an interesting observation: the lack of transfected lymphocytes in the pool of cells exposed to plasmid suggests that macrophages may have a specific receptor for free nucleic acids. Ligation of this receptor may lead to transport of DNA across the cell membrane and activation of the cell. Surprisingly the DNA is not degraded in this process and in the case of plasmid DNA may be expressed. Selective DNA uptake may be a natural scavenger function of macrophages. The macrophage would be protected in the process, since either the free DNA would be too small to be expressed, or transcription of DNA from cells leads to innocuous self-protein production.

Example 2 CONSTRUCTION OF HIV-1 *NEF* GENE UNDER THE CONTROL OF CD3 PROMOTER IN EUKARYOTIC EXPRESSION VECTOR FOR DNA VACCINE CASSETTE

Introduction:

The HIV-1 *nef* gene open reading frame is located at the 3' end of the viral genome, partially overlapping the U3 region of the 3' LTR. This gene is expressed via multiply spliced transcripts and encodes a 27 Kda cytoplasmic protein. The *nef* gene has been shown to directly influence the propagation of HIV-1 by its positive effect on the infectivities of progeny virions. Downregulation of CD4 surface antigen in established T cell lines that express the *nef* gene of HIV-1 isolates suggests that *nef* has a potential to perturb the normal function of T cells. With these functions considered together, the attenuated *nef* gene would be an attractive component of a DNA vaccine for HIV-1.

Methods:

The *nef* genes from HIV-1 positive patients were PCRed and 0.74 Kb fragments were cloned at the downstream of the CD3 enhance/promoter elements in the pNEZ vector (Figure 7). These constructs were confirmed by sequencing the vectors using the dideoxy terminator cycle sequencing kit purchased from Applied Biosystems, Inc., CA. These constructs were also tested for biological function by a CD4 down regulation assay by performing FACS analysis. The DNA was injected into Balb/C mice and sera from the mice were collected after 14 days and analyzed for the induction of humoral immune response by ELISA. Mice were sacrificed and the spleen weight was determined.

Results:

Earlier studies from our laboratory have shown that different HIV-1 antigens expressed under the control of CMV promoter in eukaryotic expression vector induce immune responses in murine, non-human primates and humans. Here we report that expression of *nef* gene (one of the HIV-1 antigens) expressed under the control of CD3 promoter showed the same immune response in a mouse model. Figures 8A and 8B depict the spleen size induced by *Nef* antigen expressed under the control of CMV and CD3 promoters. Figure 9 represents the antibody responses induced by *nef* in different expression vectors. It is clear from the figure that *nef* gene constructs expressed downstream of both the promoters induce the same level of immune response as DNA immunogens.

Table 1

Picornavirus Family

Genera: Rhinoviruses: (Medical) responsible for ~ 50% cases of the common cold.

Enteroviruses: (Medical) includes polioviruses, Coxsackieviruses, echoviruses, and human enteroviruses such as hepatitis A virus.

Apthoviruses: (Veterinary) these are the foot and mouth disease viruses.

Target antigens: VP1, VP2, VP3, VP4, PG

Calcivirus Family

Genera: Norwalk Group of Viruses: (Medical) these viruses are an important causative agent of epidemic gastroenteritis.

Togavirus Family

Genera: Alphaviruses: (Medical and Veterinary) examples include Sindbis viruses, RossRiver virus and Eastern & Western Equine encephalitis.

Rubivirus: (Medical) Rubella virus.

Flariviridue Family Examples include: (Medical) dengue, yellow fever, Japanese encephalitis, St. Louis encephalitis and tick borne encephalitis viruses.

Hepatitis C Virus: (Medical) these viruses are not placed in a family yet but are believed to be either a togavirus or a flavivirus. Most similarity is with togavirus family.

Coronavirus Family: (Medical and Veterinary)

Infectious bronchitis virus (poultry)

Porcine transmissible gastroenteric virus (pig)

Porcine hemagglutinating encephalomyelitis virus (pig)

Feline infectious peritonitis virus (cats)

Feline enteric coronavirus (cat)

Canine coronavirus (dog)

The human respiratory coronaviruses cause ~40 cases of common cold. EX. 224E, 0C43

Note - coronaviruses may cause non-A, B or C hepatitis

Target antigens: E1 - also called M or matrix protein

E2 - also called S or Spike protein

E3 - also called HE or hemagglutin-elterose glycoprotein (not present in all coronaviruses)

N - nucleocapsid

Rhabdovirus Family

Genera: Vesiculovirus: Vesicular Stomatitis Virus

Lyssavirus: (medical and veterinary) rabies

Target antigens: G protein

N protein

Filoviridue Family: (Medical)

Hemorrhagic fever viruses such as Marburg and Ebola virus

Paramyxovirus Family:

Genera: Parainfluenza Virus Type 1

Parainfluenza Virus Type 3
Bovine Parainfluenza Virus Type 3
Rubulavirus: (Medical and Veterinary)
Mumps virus, Parainfluenza Virus Type 2, Parainfluenza Virus Type 4, NewCastle disease virus (important pathogen in chickens)
Morbillivirus: (Medical and Veterinary)
Measles, canine distemper
Pneumonvirus: (Medical and Veterinary)
Respiratory syncytial virus

Orthomyxovirus Family (Medical)
The Influenza virus

Bunyavirus Family
Genera: Bunyavirus: (Medical) California encephalitis, La Crosse
Phlebovirus: (Medical) Rift Valley Fever
Hantavirus: Puremala is a hemahagin fever virus
Nairovirus (Veterinary) Nairobi sheep disease
Also many unassigned bungaviruses

Arenavirus Family (Medical)
LCM, Lassa fever virus

Reovirus Family
Genera: Reovirus: a possible human pathogen
Rotavirus: acute gastroenteritis in children
Orbiviruses: (Medical and Veterinary)
Cultivirus: Colorado Tick fever, Lebombo (humans) equine encephalosis, blue tongue

Retrovirus Family
Sub-Family: Oncorivirinal: (Veterinary) (Medical) feline leukemia virus, HTLVI and HTLVII
Lentivirinal: (Medical and Veterinary) HIV, feline immunodeficiency virus, equine infections, anemia virus
Spumavirinal

Papovavirus Family
Sub-Family: Polyomaviruses: (Medical) BKU and JCU viruses
Sub-Family: Papillomavirus: (Medical) many viral types associated with cancers or malignant progression of papilloma

Adenovirus (Medical)
EX AD7, ARD., O.B. - cause respiratory disease - some adenoviruses such as 275 cause enteritis

Parvovirus Family (Veterinary)
Feline parvovirus: causes feline enteritis
Feline panleucopeniavirus
Canine parvovirus
Porcine parvovirus

Herpesvirus Family
Sub-Family: alphaherpesviridue
Genera: Simplexvirus (Medical)

HSVI, HSVII
Varicellovirus: (Medical - Veterinary) pseudorabies - varicella zoster
Sub-Family - betaherpesviridue
Genera: Cytomegalovirus (Medical)
HCMV
Muromegalovirus
Sub-Family: Gammaherpesviridue
Genera: Lymphocryptovirus (Medical)
EBV - (Burkitts lympho)
Rhadinovirus
Poxvirus Family
Sub-Family: Chordopoxviridue (Medical - Veterinary)
Genera: Orthopoxvirus
Variola (Smallpox)
Vaccinia (Cowpox)
Parapoxivirus - Veterinary
Auipoxvirus - Veterinary
Capripoxvirus
Leporipoxvirus
Suipoxvirus
Sub-Family: Entemopoxviridue
Hepadnavirus Family: Hepatitis B virus
Unclassified: Hepatitis delta virus

Table 2

Bacterial pathogens

Pathogenic gram-positive cocci include: pneumococcal; staphylococcal; and streptococcal. Pathogenic gram-negative cocci include: meningococcal; and gonococcal.

Pathogenic enteric gram-negative bacilli include: enterobacteriaceae; pseudomonas, acinetobacteria and eikenella; melioidosis; salmonella; shigellosis; hemophilus; moraxella; chancroid; brucellosis; tularemia; yersinia (pasteurella); streptobacillus moniliformis and spirillum ; listeria monocytogenes; erysipelothrix rhusiopathiae; diphtheria; cholera; anthrax; donovanosis (granuloma inguinale); and bartonellosis.

Pathogenic anaerobic bacteria include: tetanus; botulism; other clostridia; tuberculosis; leprosy; and other mycobacteria. Pathogenic spirochetal diseases include: syphilis; treponematoses: yaws, pinta and endemic syphilis; and leptospirosis.

Other infections caused by higher pathogen bacteria and pathogenic fungi include: actinomycosis; nocardiosis; cryptococcosis, blastomycosis, histoplasmosis and coccidioidomycosis; candidiasis, aspergillosis, and mucormycosis; sporotrichosis; paracoccidiodomycosis, petriellidiosis, torulopsosis, mycetoma and chromomycosis; and dermatophytosis.

Rickettsial infections include rickettsial and rickettsioses.

Examples of mycoplasma and chlamydial infections include: mycoplasma pneumoniae; lymphogranuloma venereum; psittacosis; and perinatal chlamydial infections.

Pathogenic eukaryotes

Pathogenic protozoans and helminths and infections thereby include: amebiasis; malaria; leishmaniasis; trypanosomiasis; toxoplasmosis; pneumocystis carinii; babesiosis; giardiasis; trichinosis; filariasis; schistosomiasis; nematodes; trematodes or flukes; and cestode (tapeworm) infections.

Tables 3A and 3B

Table 3A: Change in percentage of PBMC expressing surface activation antigens..

Cell Subtype	CD28	CTLA-4	CD80	CD86	CD40	CD40-L
CD4 T-cell	++++	+++	+	++	ND	+++
CD8 T-cell	++++	+++	++	++	ND	+++
B-Cells	ND	ND	+	++	+	ND
Macrophages	ND	ND	++	++	+	ND
NK-Cells	ND	ND	-	+	ND	ND

Table 3B: Change in percentage of splenocytes expressing surface activation antigens.

Cell Subtype	CD28	CTLA-4	CD80	CD86	CD40	CD40-L
CD4 T-cell	+	+	+	+	ND	+
CD8 T-cell	+	+	+	+	ND	+
B-Cells	ND	ND	+ +	++	++	ND
Macrophages	ND	ND	+	+	+	ND
NK-Cells	ND	ND	-	+	ND	ND

(ND) Not Done; (-) 0-3% increase; (+) 3-5% increase;

(++) 5-10% increase; (+++) 10-15% increase; (++++) 15-20% increase.